Decreased Immunoglobulin Deposition in Tumors and Increased Immature B Cells in p53-null Mice


Abstract
Recent studies have hinted that there may be a relationship between p53 and the immune response. In preliminary experiments. we found significantly decreased levels of immunoglobulin deposition in 13 of 16 p53-null tumors compared with 2 of 17 tumors derived from p53-/- mice. We further explored the effect of p53 on B-cell development and function. p53-null mice contained more splenic white pulp and more immature B cells in the bone marrow compared with p53-/- mice. p53-null B cells were hyperresponsive to proliferative challenge but were not more resistant to signal-induced apoptosis. Several p53 DNA-binding sites were localized to the regulatory regions of immunoglobulin heavy and light chain genes, including the II site, which serves as an enhancer. For rearrangement of the mouse k chain D cluster genes. Levels of p53 protein and the k chain sterile transcript increased after exposure of pre-B cells to the DNA damaging agents etoposide and Adriamycin. Our observations suggest that p53 may be involved in B-cell maturation and may relay certain stress signals to affect B-cell function.

Introduction
The p53 tumor suppressor protein is a transcription factor that regulates growth arrest and apoptosis pathways in mammalian cells (reviewed in Ref. 1). p53 target genes include cell cycle regulators (p21WAF1/CIP1, MDM2, and GADD45), apoptosis inducers (bax and FAS/Apo1), and secreted proteins (Thrombospondin 1, GD-AIF, and IGF-BP3). Recent studies have suggested a possible relationship between p53 and the immune response. p53 induction was correlated with differentiation and k light chain gene expression in γ-irradiated pre-B cells (2). In addition, it has been possible to partially rescue VDJ3 recombination and T-cell development in SCID mice that are p53-/- but not p53-/- (3).

While investigating p21 expression as a marker of p53 status, we found evidence for decreased immunoglobulin deposition in tumors derived from p53-/- versus p53-/- mice. To further explore the relationship between p53 and this immunoglobulin deposition, we investigated B-cell maturation and responsiveness in p53-null and p53-/- mice. We also searched for evidence for direct p53 regulation of VDJ rearrangement. We found that p53-null mice contain increased splenic white pulp, increased numbers of immature B cells in their bone marrow, and B cells that are hyperresponsive to proliferative challenge. We identified several p53 DNA-binding sites in the regulatory regions of immunoglobulin heavy and light chain genes, including the II site recently shown to be important for rearrangement of the k light chain genes (4) and the DG52 region important for heavy chain gene rearrangement (5). p53 bound to these putative sites, and we detected induction of sterile (germ line) transcription at the k chain and Iκ loci after p53 induction. Our observations raise the possibility that p53 may be involved in B-cell maturation, a role that has implications with respect to normal and antitumor immune function, as well as the high prevalence of lymphoid malignancies in p53 knockout mice.

p53 binding to immunoglobulin regulatory sites suggests the possibility that p53 may serve as a relay of certain stress signals to affect B-cell function.

Results
Analysis of Tumors from p53-/- and p53-/- Mice. Our initial studies used tumors derived from p53-/- and p53-/- mice to examine p21 expression in tumors as a marker of p53 status. We raised antibodies that specifically and sensitively recognized mouse p21 (Fig. 1). Three anti-mouse p21 mAbs (Fig. 1A–C) and a rabbit polyclonal antibody (Fig. 1D)

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3 The abbreviations used are: VDJ, Variable Diversity Joining segments of immunoglobulin gene family; slgM, surface Immunoglobulin class M; LPS, lipopolysaccharide; GST, glutathione S-transferase; EMSA, electrophoretic mobility shift assay; FACS, fluorescence-activated cell sorting; RT, reverse transcription.
Fig. 1. A-D, reactivity of anti-mouse p21\(^{WAF/CIP1}\) antibodies. Western blot analysis of lysates derived from human GM cells incubated in the absence (Lane 1) or presence (Lane 2) of dexamethasone, or lysates derived from mouse M3 cells incubated at 37°C (Lane 3) or 32°C (Lane 4) were immunoblotted with either anti-mouse p21 mAb 23A (A), anti-mouse p21 mAb 75A (B), anti-mouse p21 mAb 65A (C), or anti-mouse p21 polyclonal sera OS100 (D). +, presence of wild-type p53 protein. Protein molecular weight size markers are indicated to the right of each panel (in thousands). The predicted mobility of p21 is indicated by an arrow to the left of A, E, analysis of tumors from p53\(^{+/−}\) and p53\(^{-/-}\) mice using an anti-mouse p21\(^{WAF/CIP1}\) mAb. Western blot analysis of a subset of the tumors shown in Table 1 was performed using anti-mouse p21\(^{WAF/CIP1}\) mAb 75A as described in "Materials and Methods." The p53 genotype of the mice from which the tumors were derived is indicated above each lane. +/−, one wild-type p53 allele and one null allele; −/−, two null p53 alleles. Protein molecular weight size markers (in thousands) are indicated by arrows to the left of the figure. The identity of each tumor shown in this figure can be found in Table 1 (Lane 1, tumor 967; Lane 2, tumor 1337; Lane 3, tumor 1005; Lane 4, tumor 1087; Lane 5, tumor 1239; Lane 6, tumor 1280; Lane 7, tumor 1367; Lane 8, tumor 1687; Lane 9, tumor 1706; Lane 10, tumor 1334; Lane 11, tumor 1640; and Lane 12, tumor 1683).

recognized a \(M, 20,000\) band by Western blot analysis of M3 murine lymphoma cells (6) at 32°C (Fig. 1A–D, Lane 4), but not at 37°C (Lane 3). The M3 lymphoma cells express a temperature-sensitive p53 gene (6) that adopts a transcriptionally active "wild-type" conformation at 32°C, but is functionally mutant at 37°C. One of the monoclonal antibodies (22A; Fig. 1A) and the polyclonal antibody (OS100; Fig. 1D) also showed reactivity against human p21 protein (Lane 2) in GM cells treated with dexamethasone to induce p53, but not in untreated GM cells (Lane 1). Cross-reacting protein bands were detected but were least prominent when mAb 75A (Fig. 1A) was used for immunoblotting (compare with Fig. 1B–D). These four antibodies also immunoprecipitated p21 and associated cyclin-dependent kinase 2 protein from M3 cells at 32°C, but not at 37°C (data not shown).

In a blinded fashion, we used the anti-mouse p21 antibodies to immunoblot 19 tumors derived from p53\(^{+/−}\) (\(n = 10\)) and p53\(^{-/-}\) (\(n = 9\)) mice. The 19 tumors (see Table 1) included a representative sampling of neoplasms, which spontaneously arise in p53-deficient mice (7). The monoclonal antibodies recognized a slower migrating band (\(M, \sim 25,000\)) in several tumors (Fig. 1E), but no band at \(M, 21,000\), leading us to suspect a posttranslationally modified p21 in vivo. The \(M, 25,000\) band was not recognized by anti-p27 antibodies (data not shown). We found no evidence for interaction between the \(M, 25,000\) band and either cyclin-
The anti-mouse p21 OS100 polyclonal antibody, when used at a titer of 1:1000, detected a doublet at M₁, 21,000-22,000 in most of the original tumors derived from p53<sup>−/−</sup> and p53<sup>−/−</sup> mice (Fig. 3). We found a poor correlation between p21 expression and p53 genotype; i.e., one of two tumors that highly expressed p21 (Fig. 3, Lanes 2 and 9) was p53<sup>−/−</sup>, and in the tumors that expressed p21 at lower levels (e.g., Fig. 3, Lanes 1, 3-7, and 10-12), there was no apparent difference between the level of p21 expression in tumors derived from p53<sup>−/−</sup> and p53<sup>−/−</sup> mice. Therefore, at least for the case of tumors derived from p53<sup>−/−</sup> and p53<sup>−/−</sup> mice, p21 expression levels did not correlate with p53 status.

**Reactivity of Mouse Sera against Tumor Antigens.** Immunohistochemical staining of two immunoglobulin-positive and two immunoglobulin-negative tumors (Fig. 4A) revealed that some immunoglobulin was extracellular, possibly associated with cellular fragments, some was associated with tumor cells, and some with rare small round cells. The precise origin of the tumor-associated immunoglobulin or the target of this immunoglobulin deposition was not clear.

We found no differences in the levels of serum immunoglobulin between p53<sup>−/−</sup>, p53<sup>−/−</sup>, and p53<sup>−/−</sup> mice (data not shown). We investigated the reactivity of sera derived from wild-type p53-containing versus p53<sup>−/−</sup> tumor-bearing mice against tumor antigens. Fig. 4B, a-c, shows results of immunoblotting a tumor derived from a p53<sup>−/−</sup> mouse and two tumors derived from p53<sup>−/−</sup> mice with sera derived from each of the same three tumor-bearing mice. One of the sera (Fig. 4Bc) from the tumor-bearing mouse whose tumor specimen is shown in Lane 2 strongly recognized novel antigens (M₁, ~35,000 and ~75,000-80,000 proteins) in the same tumor, but not in the tumor derived from a p53<sup>−/−</sup> mouse (Lane 1) or another p53<sup>−/−</sup> mouse (Lane 3). These antigens were not recognized by sera from another p53<sup>−/−</sup> tumor-bearing mouse, whose tumor is shown in Lane 3 (Fig. 4Bb), and at least the M₁, ~35,000 band was not recognized in Lane 2 when probed by sera from a p53<sup>−/−</sup> mouse whose tumor is shown in Lane 1 (Fig. 4Ba).

Fig. 4B, d and e, shows that pooled sera from p53<sup>−/−</sup> Wnt1 tumor-bearing mice recognized several protein bands (range, M₁, ~70,000-150,000) in tumors derived from either wild-type p53-expressing or p53-null mice (Fig. 4Be; arrows), whereas pooled sera from p53<sup>−/−</sup>/Wnt1 tumor-bearing mice had substantially reduced reactivity (Fig. 4Bd).

**Increased Immature B Cells in p53-null Bone Marrow.** Lymphoid tissues (spleen, mesenteric lymph nodes, and bone marrow) were harvested from four p53<sup>−/−</sup>, two p53<sup>−/−</sup>, and four p53<sup>−/−</sup> 6-week-old mice. No significant differences were noted in the gross appearance or weight of the organs. Histological examination revealed an architectural difference with increased white pulp and relatively decreased red pulp in p53-null splenic tissue (Fig. 5A, a-c). We found no differences in the numbers of B and T lymphocytes in the spleen and lymph nodes in these groups of mice (data not shown). Although similar numbers of cells were recovered from the bone marrow of p53<sup>−/−</sup>, p53<sup>−/−</sup>, and p53<sup>−/−</sup> mice, the bone marrow of p53<sup>−/−</sup> mice contained a higher percentage of pre-B cells (B220<sup>+</sup> slg<sup>M</sup>−) compared with p53<sup>−/−</sup> mice (Fig. 5B, a and b). The percentages of B cells (B220<sup>+</sup> slg<sup>M</sup>−) were

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**Table 1** Predicted p53 status of tumors derived from p53<sup>−/−</sup> and p53<sup>−/−</sup> mice

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<tr>
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<sup>a</sup> wt, wild-type; --, non-wild-type.

Dependent kinase 2 or proliferating cell nuclear antigen (data not shown). We also found no evidence that the M₁, 25,000 band was a hyperphosphorylated form of p21 (data not shown). Thus, it would appear that the M₁, 25,000 protein is neither structurally nor functionally related to p21. However, based on the expression level of the M₁, 25,000 protein, we correctly predicted the p53 genotype of 15 of 19 mice (Table 1). Since the M₁, 25,000 band intensity appeared helpful in predicting p53 genotype, we further attempted to identify it.

**Decreased Immunoglobulin Deposition in p53-null Tumors.** Analysis of several tumors with high levels of the M₁, 25,000 band and several tumors with little or no detectable M₁, 25,000 protein revealed that the M₁, 25,000 protein was immunoglobulin light chain (Fig. 2A, Lanes 1-3 versus Lanes 4-6). In tumors in which the M₁, 25,000 protein was easily detected, a M₁, 50,000 band was also easily detected (Figs. 2A, Lanes 1-3, and 1E), likely corresponding to immunoglobulin heavy chain. Five tumors derived from p53<sup>−/−</sup> mice contained higher levels of both immunoglobulin k and l chains compared with five tumors derived from p53<sup>−/−</sup> mice (Fig. 2B, compare Lanes 1-5 with Lanes 6-10). Thus, both k and l chains appear to be deposited at decreased levels in p53-null tumors. We checked whether tissue origin of the tumor could account for the difference in immunoglobulin deposition. We found examples of lymphomas that overexpressed immunoglobulin when obtained from a p53<sup>−/−</sup> mouse, whereas other lymphomas had relatively low levels of immunoglobulin if they arose in p53<sup>−/−</sup> mice (Table 1).

We reproduced the difference in immunoglobulin deposition between wild-type p53-expressing tumors and p53-null tumors by examining breast cancers obtained from wnt1 mice crossed with either p53<sup>−/−</sup> or p53<sup>−/−</sup> mice (8). Fig. 2C shows that immunoglobulin light chain deposition was observed in 6 of 7 tumors derived from p53<sup>−/−</sup>/Wnt1 mice, but not 6 of 7 breast tumors derived from p53<sup>−/−</sup>/Wnt1 mice (Table 2).
comparable between the two groups of mice. Consistent with the increased percentage of pre-B cells, bone marrow from p53−/− mice contained an increased percentage of cells expressing high levels of the heat-stable antigen, a protein expressed at high levels on pre-B and immature B cells (Ref. 9; data not shown). Frequencies of S7+ cells were not changed in the p53−/− bone marrow.

**Mitogenic Responses of p53−/− versus p53+/− B Cells.** To examine a potential relationship between p53 status and the ability of B lymphocytes to enter the cell cycle, we cultured the lymphocytes with different mitogens and measured S-phase entry by uptake of [3H]thymidine into newly synthesized DNA (Fig. 5C). Ligation of slgM using F(ab')2 fragments of polyclonal anti-IgM antibody induces mature B cells to reenter the cell cycle (10). When cultured with increasing concentrations of F(ab')2 fragments of anti-IgM antibody, splenic B cells from p53−/− synthesized significantly more DNA compared with splenic B cells from either p53+/− or p53−/+ mice (Fig. 5C). This increased proliferative response could not be attributed to increased expression of slgM by p53−/− B cells. Splenic B cells from all three groups of mice expressed comparable levels of slgM, slgD, heat-stable antigen, and the B cell-specific form of CD45 (B220) as determined by flow cytometry (data not shown).

The increased proliferative response of p53−/− B cells was not specific for slgM ligation. When cultured with the polyclonal mitogen LPS, p53−/− splenic B cells synthesized significantly more DNA compared with splenic B cells derived from either p53−/+ or p53+/+ mice (Fig. 5C).
The increased proliferative responses of B lymphocytes from the lymph nodes of p53−/− mice could not be attributed to increased numbers of B cells in p53−/− lymph nodes, because the percentages of lymph node B lymphocytes were comparable between all groups of mice (data not shown). In addition, we found no evidence that the increased proliferative responses of p53−/− B cells could be explained by a decreased apoptotic fraction when compared with p53+/+ cells (data not shown). The results from Fig. 5C suggest that B lymphocytes derived from p53−/− mice are significantly more responsive to mitogenic stimulation, synthesizing more RNA (data not shown) and DNA in response to B-cell polyclonal stimuli.

**p53 DNA-binding Sites in the Regulatory Regions of Immunoglobulin Genes.** To further explore a potential relationship between p53 and regulation of immunoglobulin gene expression or immune function, we searched for p53 DNA binding sites in the regulatory regions of immunoglobulin genes. GenBank searches identified two potential p53 DNA consensus sites in both the human DQ52 region important for heavy chain rearrangement (4) and the human VH6 gene promoter region important for regulation of immunoglobulin chain transcription (11). Analysis of the corresponding DQ52 region in the mouse revealed the presence of at least two potential p53 sites, suggesting that regulation by p53 may be conserved. In addition, we noticed a remarkable similarity of the KI site (4) to the p53 recognition element.

We tested the candidate sites for their ability to bind to p53 protein in vitro (Fig. 6A). Fig. 6A demonstrates a mobility shift of a p53 binding site from p21 (Lanes 2), as well as mobility shifts of homologous consensus p53 binding sites from the immunoglobulin gene regulatory regions KI (Lane 6), DQ52 (Lanes 8 and 10), and VH6 (Lane 12). No mobility shifts were evident in the absence of baculovirus p53 protein (Fig. 6A, Lanes 1, 3, 5, 7, 9, and 11). Competition experiments showed that a high-affinity p53 binding site derived from the p21 gene could compete with p53 binding to the KI site (Fig. 6B, Lanes 3 and 4), whereas a non-p53 binding site was a much weaker competitor (Fig. 6B, Lanes 5 and 6).

**Correlation of p53 Induction with Sterile Transcription.** Before the rearrangement of immunoglobulin genes, non-coding transcripts initiating upstream and extending beyond potential rearrangement sites are produced, spliced, and polyadenylated. The expression of the sterile transcripts Iγ and μ2, derived from the heavy chain locus and the light chain locus, respectively, may be a necessary prerequisite for immunoglobulin gene rearrangement (12-15). To determine whether p53 binding might regulate the expression of sterile transcripts and thus B-cell development, Ba/F3 (early B-cell progenitor cell line), HAC6 (pro-B-cell line), and μ (pre-B-cell line) were treated with the DNA damaging agent Adriamycin to induce p53. Adriamycin treatment of all three cell lines induced p53 protein (Fig. 7). In Adriamycin-treated Ba/F3 cells, the level of Iγ transcripts increased 2- to 3-fold (Fig. 7). λ light chain sterile transcripts were not expected and were not detected in Ba/F3 cells due to their early stage of B-cell development. Adriamycin treatment of both HAC6 and μ cells resulted in the induction of the λ germ line sterile transcripts, but not Iγ transcripts, which were already present at elevated levels.

**Discussion**

Our experiments provide a novel link between p53 and the immune response, and suggest the following model. Conserved p53 DNA binding sites located in the regulatory regions of immunoglobulin heavy and light chain genes may enhance sterile transcription and immunoglobulin gene rearrangement, which could facilitate B-cell maturation. Previous studies have implicated a role for p53 in B-cell maturation (2, 16-18). Host deficiency in p53 may lead to less efficient immunoglobulin gene rearrangement, abnormal B-cell maturation, and decreased tumor-specific immunoglobulin. Abnormal B-cell maturation in the context of complete p53 deficiency may predispose the host to lymphoid malignancy and infection. A significant number of p53−/− mice die due to infectious abscesses, in the absence of evident tumor (19).

It has been shown previously that exposure of pre-B cells to ionizing radiation induced differentiation and κ chain gene expression (2). We would propose that binding of p53 to the KI site upstream of the mouse κ chain J cluster genes may facilitate the differentiation of B cells and the production of κ chain after DNA damage. The results showing induction of germ line κ chain expression after p53 induction (Fig. 7B) are consistent with this hypothesis. Future experiments will determine whether p53 is required for the DNA damage-induced sterile transcription.

It is not clear that the decreased immunoglobulin deposition in p53-null mice is due to a loss of an antitumor response, and the precise origin of the immunoglobulin within the tumors of p53−/− mice is unknown. We speculate that plasma cells whose normal maturation was stimulated by the tumors may secrete immunoglobulin, which is then deposited within the tumors. The immunoglobulin deposition may recruit other effectors with the capacity to eliminate tumor cells. We suspect that some of the immunoglobulin we found
associated with cellular fragments may represent residue of dying cells. However, the deposited immunoglobulin did not appear to be specific for such fragments because some was associated with tumor cells and some with rare small round cells.

It is also not clear if less efficient immunoglobulin gene rearrangement in the host is the underlying mechanism of decreased immunoglobulin deposition in p53-deficient tumors. Because our initial studies involved the p53 status of the host mice, we favor a host factor to explain the differences in immunoglobulin deposition. It is interesting that one of the mouse tumors (1337) from a p53+/− mouse acquired a second hit in the p53 gene with a resultant p53−/− tumor genotype. The relatively high levels of immunoglobulin in this tumor are consistent with the hypothesis that a host factor may be responsible for the difference in immunoglobulin deposition between tumors from p53+/− and p53−/− mice. Our observation that sera derived from wild-type p53-containing tumor-bearing mice cross-reacted with antigens from tumors derived from both p53+/+ and p53−/− mice also correlates the p53 status of the host to the presumptive immune response.

It is well known that in ataxia telangiectasia there is defective VDJ recombination, immune deficiency, and an increased incidence of lymphoid malignancy (20). It has been shown that p53 induction after exposure of AT cells to DNA damaging agents is abnormally delayed (21). Therefore, it is conceivable that the defect in VDJ recombination may be due to abnormal p53 function in AT cells, i.e., a downstream effect of abnormal ATM signaling (22). The ability of p53 to bind to regulatory regions of immunoglobulin genes, which are critical for VDJ recombination, coupled with the increased numbers of immature B cells in p53−/− mice, is consistent with the possibilities that p53 may regulate VDJ recombination and that defects in such regulation may underlie certain defects in ataxia telangiectasia. Future studies will examine the effects of replacement of p53 on VDJ recombination and maturation of B cells derived from AT patients.

Because p53−/− mice appear to be predisposed to infectious disease (19), it is unlikely that the p53 status of the host is only important after tumors have developed. In addition, the high prevalence of lymphoid malignancy in p53−/− mice has not been adequately explained. We would propose that the absence of p53 contributes to a B-cell maturation defect that may then predispose p53-null mice to developing lymphomas.

Materials and Methods

Cell Lines and Culture Conditions. The human glioblastoma cell line GM was a gift from W. Edward Mercer (Thomas Jefferson University). GM cells were maintained in culture as described previously (23). Induction of the exogenous mouse mammary tumor virus promoter-driven wild-type p53 gene in GM cells was accomplished by incubating them in the presence of 1 μg dexamethasone for 20 h as described previously (24). M3 murine lymphoma cells were maintained in culture as described previously (6). Temperature shift to 32°C, to induce the wild-type conformation of the exogenous p53 temperature-sensitive mutant, was carried out for 24 h before harvesting the cells as described previously (25). The murine cell lines μ (26), HACA (27), and Ba/F3 (Ref. 28; a gift from Richard J. Jones, Johns Hopkins University, Baltimore, MD) were cultured as described previously and treated with 0.2 μg/ml Adriamycin for 20 h before harvesting.

Anti-Mouse p21WAF1/CIP1 Antibodies. GST-muWAF1/CIP1 fusion protein, containing amino acids 1-164 of p21WAF1/CIP1, was expressed in Escherichia coli after induction with isopropyl-1-thio-β-D-galactopyranoside for 2 h at 37°C. Cells were lysed by passage through a French Pressure Cell (SLM Instruments), and the insoluble GST-muWAF1/CIP1 was recovered by centrifugation. Approximately 10 mg of insoluble GST-muWAF1/CIP1 protein was resuspended in 2.5 ml of SDS-PAGE sample buffer and applied to a Bio-Rad PrepCell for purification. Eluted fractions from the PrepCell containing GST-muWAF1/CIP1 protein were pooled and dialyzed against 1× PBS overnight at 4°C. At this point, the protein remained soluble and was further concentrated using an Amicon Stir Cell.

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4 L. A. Donehower, unpublished observations.
Fig. 4. A, immunohistochemical staining of tumors derived from p53<sup>-/-</sup> and p53<sup>+/+</sup> mice. a-d, antibodies directed against murine IgG were used to stain tumors derived from p53<sup>-/-</sup> (a and b) and p53<sup>+/+</sup> (c and d) mice. The identity of each tumor shown can be found in Table 1 (a, tumor 1239; b, tumor 1337; c, tumor 1280; d, tumor 1706). B, reactivity of wild-type p53 or p53<sup>-/-</sup> host sera against tumor antigens. a-c, tumor lysates were prepared from spontaneously arising tumors in a p53<sup>-/-</sup> mouse (Lane 1), or two different p53<sup>-/-</sup> mice (Lanes 2 and 3) and immunoblotted using sera derived from the same mice (sera in A: tumor in Lane 1; sera in B: tumor in Lane 3; sera in C: tumor in Lane 2) as described in “Materials and Methods.” The p53 genotype of the mice from which the tumors were derived is indicated above each lane. Protein molecular weight size markers (in thousands) are indicated by arrows to the left of each panel. d and e, Western analysis of p53<sup>-/-</sup>/Wnt1 and p53<sup>+/+</sup>/Wnt1 tumors using pooled sera derived from either p53<sup>-/-</sup>/Wnt1 (D) or p53<sup>+/+</sup>/Wnt1 (E) tumor-bearing mice. The identity of each tumor shown can be found in Table 2 (Lane 1, tumor 2; Lane 2, tumor 10; Lane 3, tumor 30; Lane 4, tumor 70; Lane 5, tumor 75; Lane 6, tumor 98; Lane 7, tumor 154; Lane 8, tumor 162; Lane 9, tumor 372; Lane 10, tumor 388B; Lane 11, tumor 430; Lane 12, tumor 455; Lane 13, tumor 460; Lane 14, tumor 477).
For generation of rabbit polyclonal antibodies, New Zealand white rabbits were injected intranodally with 50 μg of purified GST-muWAF1/CIP1 on five successive occasions. Rabbit sera were initially screened by capture ELISA as described (29) and subsequently purified over Protein A Agarose (Pharmacia, Uppsala, Sweden).

For mouse monoclonals, female (BALB/c × C57B1/6) F1 mice (Charles River Breeding Laboratories) were immunized by i.p. injection with 10 μg of electroeluted GST-muWAF1/CIP1 protein in Ribi adjuvant (Ribi Immunocohno Research, Inc.) at weeks 0, 4, 7, and 15. Serum was obtained from the intraorbital plexus on weeks 9 and 16. Serum titers were measured by capture ELISA using GST-muWAF1/CIP1 or GST-huMDM2 as described previously (30). Specificity toward p21WAF1/CIP1 was determined by immunoblot using a 1:500 dilution of serum on purified GST-WAF1/CIP1 and on lysates of mouse fibroblasts expressing p21WAF1/CIP1. p21WAF1/CIP1 was induced in mouse fibroblasts by treatment of cells with actinomycin D at 0.5 μM final concentration for 48 h followed by lysis in Lane's lysis buffer with protease inhibitors. Antigen-antibody complexes were detected by incubation with horseradish peroxidase-conjugated goat antimouse IgG heavy and light chain (Pierce, Rockford, IL) followed by development with enhanced chemiluminescence (Amersham, Amersham Place, England) and exposure of blots to X-ray film. Candidate hybridomas were subcloned twice by limiting dilution. Monoclonal antibodies were purified from mouse ascites by chromatography over Protein G Plus-Agarose (Oncogene Research Products, Calbiochem, Cambridge, MA).

Mice, Sera, and Tumor Samples. Healthy 4-6-week-old female p53+/+, p53−/−, and p53−/− transgenic mice were obtained from Taconic Farms (Germantown, NY). The mice were euthanized using an approved Institutional Animal Care and Use Committee Protocol, which followed recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. Spleens, intestines, and thymuses were snap frozen in liquid nitrogen and kept at −80°C until further use. Sera from normal or tumor-bearing mice were stored at −80°C until used. Tumors were harvested from tumor-bearing mice (10 p53−/−, 0 p53−/−, 7 p53−/−/wnt1, and 7 p53−/−/wnt1) and stored at −80°C until protein lysates were prepared, or until cryosectioning as described previously (30). We cryosectioned and performed H&E staining of the tumors to confirm their neoplastic origin and to assure that the samples were composed primarily (>95%) of cancer cells versus infiltrating cells or stromal elements (data not shown).
Fig. 6. Analysis of p53 binding to putative DNA consensus sites in the regulatory regions of immunoglobulin genes. A, EMSA results showing p53 binding to a p21 site (Lane 2), a nonspecific site (DC5; Lane 4), a Kil site (Lane 6), DGS2 sites (Lanes 8 and 10), and a VH6 site (Lane 12). Lanes 1, 3, 5, 7, 9, and 11 contain no baculovirus p53 protein. B, EMSA results demonstrating p53 binding to a Kil site (Lane 2) and competition by increasing concentration (10 x and 20 x) of a p21 site (Lanes 3 and 4) or a nonspecific competitor (Lanes 5 and 6).

Western Blot Analysis. Equivalent amounts of protein from mouse tumors or cell lysates were separated by SDS-PAGE using 15% resolving gels, transferred to Immobilon P membrane (Millipore, Bedford, MA), and immunoblotted with either p21 or p21-H3(1-CP) antibodies (see above), anti-mouse IgG (heavy and light) antibodies (Pierce), or polyclonal mouse or chain-specific antibodies (Cappell, Durham, NC) as described previously (25).

EMSAs. The mouse anti-human p53 monoclonal antibody pAb421 (Ab1; Oncogene Research Products, Calbiochem) was used to detect specific DNA binding by p53 using EMSAs as described previously (31), except that either a p21 binding site was used as the probe (5'-CAGGAAACATGGCCAACATGGGACC-3'; site 1; Ref. 30), or putative p53 sites from immunoglobulin regulatory regions were used (Table 3). The DNA-binding data contained the following in 20 μl: 10% glycerol, 20 mM HEPES (pH 7.5), 25 mM KCl, 2 mM DTT, 2 mM MgCl₂, 0.2% NP40, 1 μg poly[(deoxy)nuclease-[(deoxy]cytidylic acid], and 2 μg of baculovirus-produced wild-type p53 (32) (gift from B. Vogelstein, Johns Hopkins University). The cell lysis buffer was prepared as described previously (33), except that it also contained 1% antipain (Sigma, St. Louis, MO), 1% leupeptin (Sigma), 1% phenylalanine A (Sigma), 1% chymostatin (Sigma), and 0.1% AEBUF (Oncogene Research Products, Calbiochem).

Stimulation of B-Cell Proliferation. LPS (from Salmonella typhosa) was purchased from Sigma. Rabbit antibody specific for mouse IgM was purified from the sera of rabbits immunized with mouse IgM by chromatography on protein A-Sepharose (Sigma). The purified immunoglobulin was digested with papain to generate Fab' fragments as described (34). Bone marrow cells were flushed from the femurs and tibias of individual p53⁻/⁻ and p53⁺/⁻ mice. Red blood cells were lysed by osmotic shock in Gey's solution (35). Mature B cells were isolated from the spleens of individual p53⁻/⁻, p53⁺/⁻, and p53⁻/⁻ mice exactly as described (36). Lymph node cells were isolated from pooled axillary, brachial, inguinal, and mesenteric lymph nodes of individual p53⁻/⁻, p53⁺/⁻, and p53⁻/⁻ mice. B cells (2 x 10⁵/well) were cultured in B-cell assay medium (RPMI 1640 with 10% FCS, 0.1 mM nonessential amino acids, OPI (Life Technologies; proprietary formula), 100 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg 2-mercaptoethanol) with either Fab' or fragments of rabit anti-mouse IgM, or with 50 μg/ml LPS in 96-well flat-bottom plates (Costar). To measure entry into S phase, [3H]thymidine (1 μCi/well) was included for the final 12 h of a 72-h culture. Lymph node cells (2 x 10⁵/well) were cultured with either Fab' fragments of rabbit anti-mouse IgM, or with concanavalin A in 96-well flat-bottom plates. To measure entry into S phase, [3H]thymidine (1 μCi/well) was included for the final 12 h of culture. Thymidine incorporation was determined by liquid scintillate spectrometry.

FACS. Phycoerythrin-conjugated antibody specific for mouse CD45R (B220) and phycoerythrin-conjugated antibody specific for mouse Thy-1.2 were purchased from PharMingen (San Diego, CA). FITC-conjugated goat Fab'(ab')₂ antibody specific for mouse IgM was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Cells (5 x 10⁵/10⁴/μl group) were equilibrated in staining buffer (PBS with 0.1% BSA, 0.1% NaN₃) for 30 min on ice. Antibody was added (1 μg/ml in staining buffer), and cells were incubated for an additional 30 min on ice. After two washes with staining buffer, cells were fixed in 1% paraformaldehyde and kept at 4°C until analyzed on a FACScan (Becton Dickinson, San Jose, CA) using Lysis II software.

RT-PCR of Immunoglobulin Sterile Transcripts. Total RNA was isolated from μ, HAC6, and Ba/F3 cells using the TOTAL RNA isolation kit (Ambion), and 5 μg was reverse transcribed using SuperScript RT II (Life Technologies) according to the manufacturer's protocol. PCR assays were carried out using one-tenth of the RT reaction in a final volume of 20 μl containing 0.1 μM 5'-3' and dCTP as described previously (37). The following primer pairs were used to amplify the μ gene transcript: 5'-GTT GGC TTT GAA GGA ACA ATC CCA C-3' and 5'-TCT GAA CCT TCA AGG ATG CTC TTT-3'. PCR was carried out for 15 or 30 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 60 s, and chain elongation at 72°C for 30 s. 28S rRNA was used as an internal control for amplification efficiency as described previously (37). Primers and conditions for α light chain germ line transcript were done as described (4).
Table 3 p53 DNA-binding sites located in the regulatory regions of immunoglobin genes

DNA sequences of Ig regulatory regions were obtained from Refs. 33, 34, 39, and 55. The highly conserved 4th cytosine and 7th guanine in each repeat sequence are highlighted in bold.

<table>
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<tr>
<th>Candidate site</th>
<th>5’ position</th>
<th>Homology to consensus p53 DNA binding site</th>
<th>3’ position</th>
<th>% homology</th>
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<td>p10-13Y</td>
<td>RRRACGYYY-3’</td>
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<td>ttcc AcCgAcTCT gaga-3’</td>
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<td>494</td>
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References


